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The expression of heat shock protein 70, heat shock protein 90 and heat shock factor 1 within the bovine midcycle corpus luteum after stimulation with temperature in vitro and PGF2alpha in vitro and in vivo

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THE EXPRESSION OF HEAT SHOCK PROTEIN 70, HEAT SHOCK PROTEIN 90 AND HEAT SHOCK FACTOR 1 WITHIN THE BOVINE MIDCYCLE CORPUS LUTEUM AFTER STIMULATION WITH TEMPERATURE *IN VITRO* AND PGF2α *IN VITRO* AND *IN VIVO*

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DEDICATION

To Matt, your love and support have been invaluable. As I close this chapter of my life I look forward to opening another one with you.

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ABSTRACT

THE EXPRESSION OF HEAT SHOCK PROTEIN 70, HEAT SHOCK PROTEIN 90 AND HEAT SHOCK FACTOR 1 WITHIN THE MID-CYCLE BOVINE CORPUS LUTEUM AFTER TREATMENT WITH HEAT IN VITRO AND PGF2A IN VITRO AND IN VIVO

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Environmental and biological stressors affect cellular function. Heat shock proteins (HSPs) are molecular chaperones that safeguard cells against these stressors by protecting endogenous proteins from degradation. Heat shock factors (HSF) are their transcriptional regulators. In the present study, we evaluated whether heat (Experiment 1) or prostaglandin F2alpha (PGF2α; Experiments 2 and 3) induces the expression of HSP 70, HSP 90 and HSF1 in the bovine corpus luteum (CL) . In Experiment 1, mid-cycle corpora lutea (day 10-11, day 0=estrus; n=4) were obtained from dairy cows, and the tissue was dissociated with collagenase. One million steroidogenic luteal cells were then seeded into T25 flasks containing Ham's F12 medium and incubated overnight at 37°C. After a medium change, cells were either maintained at 37°C or at 42°C for 10 mins, 2 hrs, or 24 hrs, respectively. Thereafter, cellular extracts were prepared for western blot analysis. In all samples, the immunoreactive bands co-

migrated with their corresponding positive controls, a HeLa cell lysate (HSP 70, HSF 1) or recombinant HSP 90 protein. The expression of HSP 70 was increased ($p<0.05$) over respective controls at 24 hours and 2 hours, and HSP 90 was increased ($p<0.05$) over respective controls at 24 hours. In Experiment 2, mid-cycle corpora lutea (day 10-11, day 0=estrus; n=4) were obtained from dairy cows, and the tissue was dissociated as described above. Steroidogenic luteal cells were treated with PGF2α (0, 10, 50, 100 or 500 µg/ml) for either 30 mins or 24 hrs. Immunoblots revealed that HSP 70, HSP 90 and HSF 1 expression did not change ($p>0.05$) for any PGF2α concentration at any time point. In Experiment 3, mid-cycle cows received saline (n=4) or a luteolytic dose of PGF2α (intramuscular; Lutalyse), and corpora lutea (n=4 for each time point) were collected 30 min and 24 hrs post injection. Tissue extracts were analyzed by western blotting. Similar to Experiments 1 and 2, HSP 70 and 90 proteins were detected in all luteal extracts. In contrast to Experiment 1, no differences ($p>0.05$) in HSP 70 expression were observed. However, HSP 90 expression was elevated ($p<0.06$) above controls 30 min post-PGF2α treatment, before returning to control levels at 24 hours. On the other hand, HSF 1 expression remained unchanged ($p>0.05$). In summary, in vitro heat treatment elicited a later response in luteal cells by increasing HSP 70 expression at 24 hrs, while in vivo PGF2α treatment leads to an earlier response by increasing HSP 90 expression at 30 min. This was not due to differential HSF 1 expression. Thus, we conclude that heat shock and PGF2α appear to induce the expression of specific HSPs in the bovine corpus luteum in a time-dependent manner.

CHAPTER I

LITERATURE REVIEW

Introduction

The ability for a species to survive depends primarily on its capacity to adapt to its surroundings and successfully reproduce. Reproduction is vital to assure the next generation. Sexually reproduction has given mammals the ability to combine sperm and egg to form an embryo. A number of hormones are required to carry the pregnancy to term. One of the most important is the steroid hormone, progesterone. Progesterone is coined 'the hormone of pregnancy' and is produced by the transient endocrine gland, the corpus luteum (Davis and Rueda, 2002). The corpus luteum, or CL, is the focus of my research and will be described in detail later.

Reproduction, as mentioned above is important for species survival. Another important characteristic is the ability to adapt to change, both within the environment, and within the body. An organism will work to maintain homeostasis. Homeostasis is one of the most remarkable and most typical properties of highly complex systems. A homeostatic system is a system that maintains its structure and functions by means of multiple processes. One such process is the production of heat shock proteins.

Heat shock proteins are produced under a variety of stimuli in order to maintain homeostasis with in a cell. Environmental conditions, such as heat as well as internal conditions such as prostaglandins are two different stimuli that elicit the response of heat shock proteins. These heat shock proteins work to restore homeostasis to the cell so that the cell can maintain normal functions.

Reproduction

The ability to reproduce has driven evolution and a species' ability to survive. Sexual reproduction gives mammals the ability to produce offspring that are a mix of paternal and maternal characteristics. This gives the advantage of genetic diversity. The focus of my research is on the bovine reproductive cycle.

Estrous Cycle

The estrous cycle consists of a series of predictable reproductive events beginning at estrus and ending at the subsequent estrus (Garverick and Smith, 1993). Cows are termed polyestrus because they have estrous cycles that occur continually throughout the year. The length of the cow estrous cycle is generally 21 days (Peters, 1987). There are two major phases to the estrous cycle, the relatively short follicular phase (3-5 days) and the longer luteal phase (15-17 days) (Garverick and Smith, 1993). The follicular phase consists of proestrus and estrus while the luteal phase encompasses metestrus and diestrus (Hansel, 1973). Before characterizing the estrous cycle at length, background information on the structure of the ovary will be provided.

Ovary

The ovary is the female gonad. It undergoes constant changes in size, structure and function (Peters, 1987). The primary function of the ovary is to produce female gametes (i.e. oocytes) and the hormones estradiol and progesterone. However, the ovary also produces oxytocin, relaxin, inhibin and

activin. Histologically, the ovary is covered by a one-cell layer thick germinal epithelium, under which lies the tunica albuginea, a tough, thick, connective tissue layer. The hilus is a structure in which blood enters and exits the ovary. Under the tunica albuginea is the ovarian cortex where the oocytes reside. These oocytes are constantly being recruited each cycle and several are picked to become primordial follicles (Wassarman, 1988).

Follicular Phase

In cows, the follicular phase comprises about 20% of the estrous cycle but follicles grow and degenerate almost constantly throughout the estrous cycle (Peters, 1987). There are generally three follicular waves over the course of the bovine estrous cycle although some cows have as few as two or as many as four. Follicular waves are stimulated by follicular stimulating hormone.

Primordial follicles advance to become primary follicles. A primary follicle is characterized by an oocyte surrounded by a single layer of follicular cells. Development continues from a primary follicle to a secondary follicle, in multiple layers of granulosa cells surround the oocytes (Wassarman, 1988). The granulosa cell compartment has no blood supply, but is separated from the vascular theca cell compartment by a basement membrane.

Tertiary follicles form from secondary follicles, and are characterized by the accumulation of fluid and the formation of an antrum. This fluid is high in sugar and estradiol and acts to nourish the granulosa cells. Not all primordial follicles mature to tertiary follicles. Only 8 to 10 primary follicles are selected to form secondary follicles and still fewer of those are recruited to become tertiary

ones during each ovarian or estrous cycle (Greenwald, 1988). The follicles progress from several tertiary follicles to one dominant Graffian follicle. The graafian follicle is the most mature follicle and contains one contiguous fluid-filled cavity. It is from this structure that the egg will soon ovulate (Garverick and Smith, 1993).

Ovulation is the process in which the oocyte is expelled from the Graffian follicle. Follicular fluid buildup paired with structural degradation of the follicular wall and inflammation trigger this process. Once the follicle ruptures and the oocyte is released. Afterwards the ruptured follicle wall collapses and a new structure called the corpus hemorrhagicum is formed. As time passes, the corpus hemorrhagicum becomes the corpus luteum, or CL (Garverick and Smith, 1993).

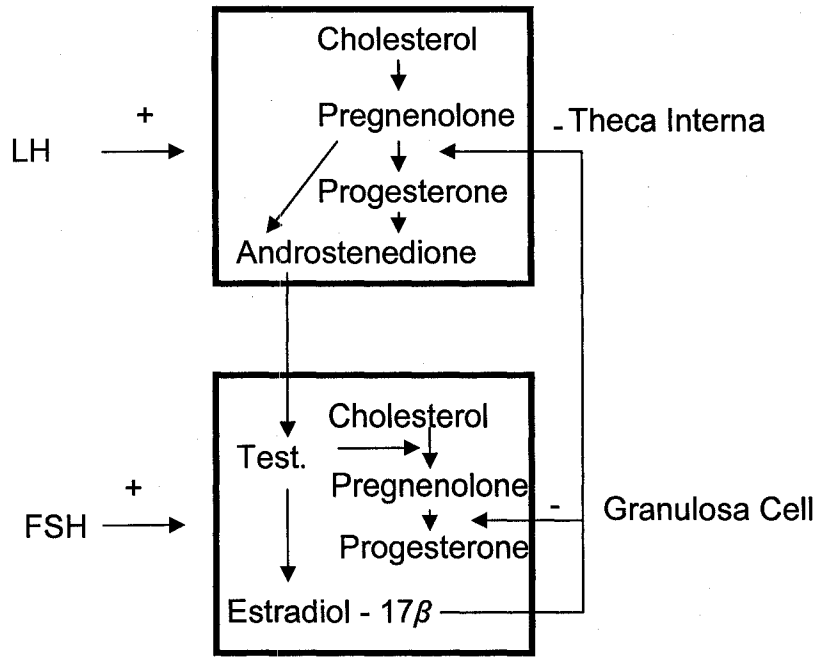
The events of the follicular phase are mediated by several hormones. Gonadotropin releasing hormone or GnRH is a decapeptide released from the hypothalamus of the brain containing terminals of neurons in the surge and tonic LH centers. GnRH secretioin causes the release of gonadotropins from the anterior pituitary: follicle stimulating hormone and luteinizing hormone. FSH or follicle stimulating hormone is secreted from the anterior pituitary in response to GnRH and promotes follicular development. Likewise luteinizing hormone (LH) is produced in the anterior pituitary upon GnRH stimulation and causes ovulation and subsequent development and maintenance of the CL (Hansel, 1973).

As a result of follicular growth and development, estradiol is produced. Estradiol is synthesized by the granulosa cells of the tertiary follicle (Dorrington et

al., 1975);(Henderson and Moon, 1979) and targets the hypothalamus and the entire reproductive tract and mammary gland. Its primary actions are to provoke sexual behavior, further stimulate secretion of GnRH, elevate secretory activity of the entire reproductive tract, enhance uterine motility and induce estrus. One of its most important roles is to cause and maintain female secondary sexual characteristics. The production of estradiol is complicated in that it requires two cell types, the theca and granulosa cells. One of the first descriptions of the two-cell theory was in the mare (Short, 1962), where follicular fluid and luteal tissue were studied. An even earlier study (Falck, 1959) described an eloquent experiment involving transplantation of vaginal tissue to the rat cornea. The vaginal tissue served as an indicator of estradiol action. The results showed that granulosa cells on their own always grew to become luteal cells, but theca cells cornified the vaginal tissue provided that some granulosa cells were present.

It is the production of estradiol in the absence of progesterone that stimulates the release of GnRH from the hypothalamus. This surge of GnRH stimulates the release of LH and FSH, which in turn drives the production of estradiol by the Graffian follicle. In the rat, a paper by Dorrington et al. (1975) demonstrates that theca cells under the influence of LH produce androgens which are then transported to the granulosa cells for aromatization under the influence of FSH. A surge of LH lasting 10 hr occurs when estradiol reaches a threshold level, inducing ovulation 24 hours after the peak of LH (Hansel, 1973). A later article (Fortune, 1986) details the two cell theory of estradiol production by bovine follicular cells. Refer to figure on the next page.

Figure 1. Two cell theory of estradiol production by the bovine CL



This article provided evidence that theca cells supply granulosa cells with the androgens needed for estradiol production. The interaction between the theca and granulosa cells is necessary for the production of estradiol in bovine preovulatory follicles.

During the latter part of the follicular phase, the female becomes sexually receptive. This period of estrus lasts only 18-19 hours in the cow, and ovulation generally occurs soon after this (Hansel, 1973).

LUTEAL PHASE

The luteal phase starts with ovulation and ends with regression of the corpus luteum. After ovulation, the theca interna and granulosa cells undergo luteinization. Luteinization is the process in which granulosa cells and theca cells are transformed to large and small luteal cells, respectively, through the actions

of LH (McNutt, 1924;Peters, 1987);(Niswender et al., 1985). Granulosa derived large luteal cells have a limited ability to divide, while theca derived, small luteal cells retain the ability to divide and are thought to grow into large luteal cells (Donaldson and Hansel, 1965).

The young corpus luteum or the corpus hemorrhagicum has a bloody appearance due to ruptured blood vessels during ovulation. After 3-5 days the corpus hemorrhagicum changes and begins to increase in size and lose its red appearance, taking on a yellow color. This structure is now called the corpus luteum (CL), meaning yellow body. The CL is known as the transient endocrine organ of the reproductive system (Niswender et al., 1985), and its primary function is the secretion of progesterone (Garverick and Smith, 1993).

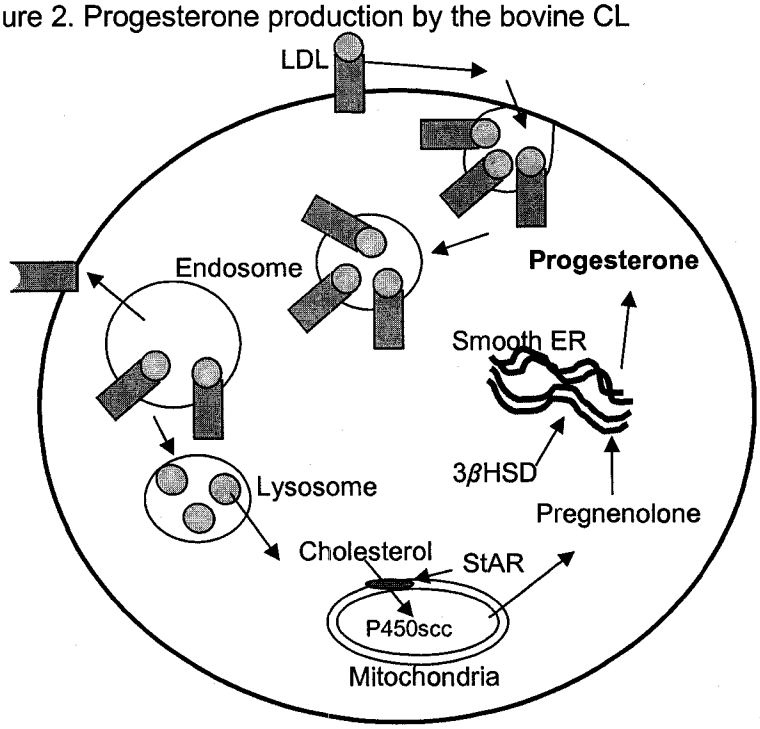
The mature CL contains two steroidogenic cells types. The small luteal cells are 8-22 μm in diameter, have lipid droplets, large quantities of smooth endoplasmic reticulum and are spindle shaped (Niswender et al., 1985). They also contain PGF2α binding sites (Chegini et al., 1991). Large luteal cells are greater than 22 μm in diameter, contain large amounts of smooth and rough endoplasmic reticulum and are more spherical in shape (Niswender et al., 1985). They contain secretory granules and also possess receptors for PGF2α. Basal production of progesterone is 10-40 fold higher in these cells than the small luteal cells (Juengel and Niswender, 1999).

The presence of LH is critical for the production of progesterone (Hansel, 1973). While both small and large luteal cells contain receptors for LH, there are fewer receptors on large luteal cells. Only small luteal cells respond to LH by

increasing progesterone production (Niswender, 2002). This is somewhat puzzling as 85% of progesterone is secreted by large luteal cells (Niswender et al., 1985). The increased levels of progesterone secreted by large luteal cells might have something to do with the availability of the P450scc enzyme complex required to make the conversion from cholesterol to pregnenolone (Niswender, 2002).

Progesterone has many functions, but it is primarily responsible for the establishment and maintenance of pregnancy, as well as ovarian cyclicity in most mammals (Short, 1967). Insufficient production of progesterone is believed to be an important contributor to reproductive failure (Liu et al., 1995).

Figure 2. Progesterone production by the bovine CL



Adapted from Niswender G.D. 2002

In order to produce progesterone, the CL requires cholesterol and LH (small cells) (Niswender, 2002). Cholesterol is carried in the form of low density lipoprotein (LDL) or high density lipoprotein (HDL) and is transported through the cell membrane via receptor mediated endocytosis (refer to figure above). Lysosomal enzymes then lyse the membrane of the endosome and release the LDL/HDL. The free cholesterol can then be transported to the mitochondria with the help of the cytoskeleton (Niswender, 2002). Cholesterol then makes its way inside the mitochondria with the help of steroidogenic acute regulatory protein (StAR). StAR transfers cholesterol to peripheral-type benzodiazepine receptors where it is transported across the membranes. Once inside the inner half of the inner membrane, of the mitochondria, using NADPH and O_2, cholesterol is converted to pregnenolone by cytochrome P450scc. Pregnenolone is then transported outside the mitochondria to the smooth ER where 3β-hydroxysteriod dehydrogenase converts pregnenolone to progesterone (refer to figure above) (Juengel and Niswender, 1999).

The primary target organs for progesterone are the hypothalamus, uterus and mammary glands (Garverick and Smith, 1993). Progesterone readies the uterus for the conceptus by stimulating maximal secretion of the endometrial glands, making an environment suitable for the attachment of the embryo. Progesterone also reduces contractions of the muscular myometrium contributing to attachment of the conceptus and suppresses maternal immune response to fetal antigens once the embryo attaches and begins to grow (McCracken, 1999).

During pregnancy progesterone reduces cyclic ovarian activity and contributes to mammary development (McCracken, 1999).

The CL is composed of a heterogeneous cell population including small and large luteal cells, fibroblasts and endothelial cells (Garverick and Smith, 1993). Angiogenesis, or the formation of blood vessels happens quickly and the CL becomes highly vascularized (Acosta et al., 2003). This blood supply is important because it gives the luteal cells proper nutrients and also carries away waste products. Certain enzymes called matrix metalloproteinases degrade surrounding tissues so that growth of blood vessels can continue in the CL (Reynolds and Redmer, 1999).

Pregnancy is required to maintain the CL and in the event that pregnancy does not take place, the CL undergoes luteolysis in which progesterone production ceases and the tissue regresses, enabling a new cycle to begin.

Luteolysis

Luteolysis results in cessation of progesterone production, structural regression of the CL to a corpus albicans, and follicular development. PGF2α is universally known as the luteolysin in most mammals (McCracken et al., 1972),(Hansel, 1973) and causes regression of the CL. PGF2α is found in many tissues, including lung extracts of sheep and pigs (Bergstrom and Samuelsson, 1962), the sheep iris (Anggard and Samuelsson, 1964) as well as the thymus, pancreas, brain and kidney (Bergstrom, 1966). Of special interest is the identification of PGF2α in menstrual fluid, as PGF2α has a large influence in the reproductive cycle of the female. PGF2α is a potent vasoconstrictor and is

present in the endometrium (McCracken et al., 1972). It is released from the uterus at the end of the luteal phase in a series of pulses and reaches the ovary through countercurrent transfer in the utero-ovarian vasculature of the broad ligament (Poyser, 1995). The pulsatile manner of release is advantageous for luteolysis (McCracken, 1999). If the CL was constantly bombarded by high levels of PGF2α, receptors would down regulate and be ineffective. The pulsatile nature of PGF2α allows the CL to remain receptive and respond to each pulse with increasing loss of structure and function. Arosh and colleagues (2004) showed that the bovine CL has the ability to autoregulate PGF2α function. Synthesis of PGF2α occurs primarily in the large luteal cells of the CL (Arosh et al., 2004).

Early studies established that the endometrium of the uterus was the site of PGF2α synthesis which is regulated by progesterone and estradiol (McCracken, 1999). Progesterone and estradiol work together to regulate oxytocin receptors in the endometrium. Estradiol enhances the formation of the receptors, while progesterone reduces their number. Oxytocin is produced by the posterior pituitary and functions to increase uterine mobility and also promotes uterine PGF2α synthesis. Oxytocin is also produced in finite amounts by the CL.

Early experiments in the guinea pig found that the uterus is essential for normal luteal regression (Loeb, 1923). After the uterus of the guinea pig was removed, the CL continued to function for sixty days, thus equaling or slightly surpassing the time span of a normal pregnancy. Later studies in the ewe and

cow (Wiltbank J.N., 1956) showed the same results. When small portions of the uterus were left behind, no delay in the return to estrus was noted. The question of what the uterus produced to cause the regression of the CL remained. McCracken et al. (1972) showed that prostaglandin F2α is the luteolytic hormone in sheep. This was shown by using sheep with a transplanted ovary attached to the jugular vein of the neck. PGF2α infused into the neck over a period of 3 to 6 hr induced luteal regression with the induction of a new estrous cycle. Because of the rapid metabolism of PGF2α by the lungs (Piper et al., 1970a);(Piper et al., 1970b) it is thought that PGF2α reaches the CL by countercurrent transfer. Since most of the ovarian artery passes by the utero-ovarian vein before entering the hilus of the ovary, it has been considered that substances might diffuse from the vein to the artery and pass directly to the ovary (Barrett et al., 1971).

Adaptation to Change

Homeostasis, or an organisms' ability to adapt to change, whether external or internal is a defining property of all living things. Temperature fluctuations, along with changes in humidity are environmental cues that affect the way an animal regulates its body processes. Adjusting to environmental changes while still carrying out physiologic functions is of paramount importance. The remainder of this literature review addresses the essential relationship between stress and fertility. Heat stress and PGF2α, a critical factor of inflammatory stress are the focus of this review.

Heat Stress

Dairy cows subjected to heat stress during the summer months in temperate zones or in the tropics often have lower fertility rates. Pregnancy rates decline from 40-60% in the winter to 10-20% in the summer (Wolfenson et al., 1997). This drop in fertility usually occurs between June and September in the northern hemisphere, and fertility rates remain low through October and November (Wolfenson et al., 2000). Obviously, any decline in fertility is a serious economic concern to the dairy industry. The hope is that by determining the cause(s) of reproductive failure, management practices could be developed to improve fertility in the dairy herds.

This reduction in fertility rates can be attributed to many different factors. Cows subjected to high temperatures, have less dry matter intake which results in a negative energy balance (West, 2003). Negative energy balance, in turn, can lead to decreased production of GnRH and LH, which results in decreased estradiol concentration. As a consequence, cows exhibit a poor estrus and have poor oocyte quality (Rensis and Scaramuzzi, 2003).

Another possible factor contributing to poor fertility is the decreased function of the CL. Heat stress can impair the function of the preovulatory follicle which in turn may affect subsequent CL function and progesterone concentration (Breuel et al., 1993). Inadequate production of progesterone can lead to a poor uterine environment and subsequent implantation failure (Rensis and Scaramuzzi, 2003).

The role progesterone plays in the fertility of heat stressed cows is somewhat contested. There are studies which report decreased (Howell et al., 1994); (Jonsson et al., 1997), increased (Trout et al., 1998;Abilay et al., 1975) or unchanged (Roman-Ponce H., 1981) concentrations of progesterone in heat stressed cows. The observed variations in progesterone concentration could be due to the nature of the heat stress (i.e. whether the heat stress is chronic or acute) and genetic factors associated with milk yield. These factors along with environmental and management practices all play a role in CL function during heat stress (Rensis and Scaramuzzi, 2003). Chronic heat exposure, like that cows experience during the summer months of tropical climates, would decrease plasma concentrations of progesterone. This is possibly associated with delayed

luteolysis (Wilson et al., 1998b) and longer estrous cycles. Diminished concentrations of progesterone also impair follicular development, leading to poor oocyte maturation and high rates of embryo death (Ahmad et al., 1995). Conversely, acute heat stress, like cows experience in a heat chamber, results in increased progesterone production by the CL (Howell et al., 1994). This increase is attributed to decreased concentrations of estradiol, which delay the late stage decline of progesterone production by the CL (Wilson et al., 1998a).

Dairy cows bred for increased milk production often have difficulty with heat stress because of the already high metabolic rates associated with the demands to produce large quantities of milk. A by-product of their metabolism is the production of heat, which when coupled with high environmental temperatures leads to infertility (Rensis and Scaramuzzi, 2003).

The species of cow may also determine the extent to which heat stress affects fertility rates. Two species of cattle, *Bos taurus* and *Bos indicus,* appear to handle heat stress differently. While *Bos taurus* had a maximum rectal temperature of 41.9 °C, whereas maximum rectal temperature in *Bos indicus* is lower at 41.2°C (Beatty et al., 2006), suggesting there is a link between genetics and susceptibility/resistance to heat stress.

One determinant of tissue and cellular susceptibility to heat stress is the expression of heat shock proteins.

Heat Shock Proteins

Heat shock proteins (HSP) aid in the cell's ability to protect itself in times of stress. They act as molecular chaperones to stabilize the three-dimensional

tertiary functional conformation of proteins without being part of the final protein structure (Ellis, 1987). Proteins that denature and lose their unique three-dimensional structure can no longer function normally. Thus, molecular chaperones are "proteins that bind to and stabilize an otherwise unstable conformer of another protein – and, by controlling binding and release, facilitate its correct fate *in vivo*" (Hartl, 1996). Chaperones do not determine the tertiary structure of the folding proteins, but help them find their structure more efficiently (Csermely et al., 1998).

The genes encoding heat shock proteins are highly conserved throughout evolution, with representatives from distant prokaryotic and eukaryotic species having at least 50% identical coding (Hunt and Morimoto, 1985). Even thermophilic organisms, whose optimum growth temperature lies between 50-90°C respond to a sudden temperature rise by rapidly increasing expression of HSP (Neuer et al., 2000). One of the earliest studies of heat shock proteins was conducted with *Drosophila* over 30 years ago. Using a light microscope, chromosomal puffing was observed after exposure to increased temperature after only 2-3 minutes (Ritossa and Vonborstel, 1964); (Ritossa, 1996). It was also noted that the normal puffing pattern of the chromosomes was reduced, signifying a reduction of protein synthesis (Schlesinger, 1989). This effect occurred quickly but puffs reached their maximum size within 30 minutes and then regressed. The experiments further showed that isolated organs were also capable of this reaction, demonstrating that the response did not require an intact organism.

Because the first discovery of this class of proteins was a result of exposure to high temperatures, they were consequently termed heat shock proteins. It is now known that a variety of cellular stressors stimulate the expression of HSPs, including ethanol, heavy metals, amino acid analogs, certain ionophores, anoxia, free radicals, ischemia (Morimoto et al., 1992), hypoxia (Benjamin et al., 1990), as well as prostaglandin F2α (Khanna et al., 1994). Over time, a large number of heat shock proteins have been discovered. Because they possess such a broad range of functions, they were named according to their molecular weight (Csermely et al., 1998). The current members are divided into six families, and are grouped according to molecular weights: i.e. HSP 100, HSP 90, HSP 70, HSP 60, small HSPs and ubiquitin (Kochevar et al., 1991). The HSPs of interest for the current study are HSP 70 and 90.

Heat Shock Protein 70

One of the most heavily studied HSPs is HSP 70. HSP 70 is generally composed of a 44 kDa amino terminal ATPase domain, an 18 kDa peptide binding domain and a 10 kDa carboxy-terminal variable domain of unknown function (Wegele et al., 2004). HSP 70 is thought to assist in 10-20% of all *de novo* folding of bacterial proteins (Bukau et al., 2000). Because average protein size in eukaryotes has increased (Mayer, 2005), it is thought this number is much higher in animals (Mayer and Bukau, 2005). HSP 70 has many roles within reproductive physiology. One such role is in rat and mouse spermatogenesis (Allen et al., 1988). Disruption of the HSP 70-2 gene results in failed meiosis,

germ cell apoptosis and male infertility in the mouse (Dix et al., 1996). HSP 70 is also important in oogenesis (Heikkila et al., 1997). Growing oocytes express high levels of the constitutive form of HSP 70, HSC 70. Later, after meiosis, synthesis of HSC 70 ceases, explaining why oocytes exhibit a high sensitivity to thermal stress. The HSP 70 terminology can often be confusing, since at least 4 and possibly as many as 8 forms exist (Kochevar et al., 1991). The inducible 72kDa form is the most studied.

HSP 70 is localized in distinct cellular compartments of the cell. In eukaryotes, HSP 70 is found in the cytosol, mitochondria, and the lumen of the endoplasmic reticulum (Wegele et al., 2004).The promoter region of HSP 70 contains multiple heat shock elements (Morimoto et al., 1992). Heat shock elements, or HSE, contain a repeated sequence nGAAn, which is the binding site for the transcription factor, heat shock factor (HSF). The role of HSP 70 is normally to aid in protein folding (Mayer and Bukau, 2005). HSP 70 binds short hydrophobic segments in partially folded polypeptides which then prevents aggregation (Wegele et al., 2004) and allows correct folding of the protein upon release. Without HSP 70, the hydrophobic surfaces of other proteins tend to interact and clump together preventing the protein from finding its unique tertiary structure. In addition HSP 70 is involved in important cellular processes, including the transport of proteins across membranes (Herrmann and Neupert, 2000) and steroid receptor functionality (Pratt et al., 2004).

Additionally, HSP 70 also has a role in the immune system, as it acts as a chaperone in cell-surface antigen presentation (Young, 1990). In this manner,

HSP 70 alerts the immune system to native cells that have irreversible damage (McPherson et al., 1993).

Heat Shock Protein 90

Heat shock protein 90 is relatively abundant in most, if not all unstressed prokaryotic and eukaryotic cells (Kochevar et al., 1991) and accounts for 1-2% of cytosolic proteins (Pratt, 1997). A mutation to HSP 90 in S. *cerevisiae* is lethal to the organism (Borkovich et al., 1989). Nearly 100 proteins are known to be regulated by HSP 90 (Pratt and Toft, 2003). Two forms exist and are know as the α and β forms. These two forms are 86% identical and are most likely the result of a gene duplication that took place about 500 million years ago (Moore et al., 1989). HSP 90β is less inducible than HSP 90α, but because the α and β forms are so similar, researchers often do not discriminate between them. In this study however, my focus is on the α form.

HSP 90 is an elongated dimer consisting of three major domains. These domains include a highly conserved amino-terminal ATPase domain, a middle domain and a carboxy-terminal dimerization domain (Wegele et al., 2004). Many cell types share similar amino acid sequences. For example, *Escherichia coli* HSP 90 has a 42% similarity in amino acid sequence to human HSP 90 (Pratt, 1997).

HSP 90 is a phosphorylated dimer; dimerization is necessary for HSP 90's functionality. HSP 90 is one of the stickiest proteins in the cytosol and acts as a kind of molecular glue in cells (Csermely et al., 1998). Like HSP 70, HSP 90

serves as a molecular chaperone to facilitate the folding of a variety of proteins and has roles in steroid hormone receptors.

HSP 90 provides a number of housekeeping functions in unstressed cells, one such function being a "cradle-to-grave" chaperone for steroid receptors (Pratt, 1997), regulating proper receptor folding, trafficking, transcriptional activation and turnover. HSP 90 is directly bound to progesterone, estradiol and glucocorticoid receptors (Rexin et al., 1988) and is required for the formation of glucocorticoid receptors. However, it is not required for high affinity ligand binding conformation with progesterone and estradiol.

HSP 90 also has novel roles outside the cell. Recently, a study implicated a connection between HSP 90 and matrix metalloproteinases. Eustace et al. (2004) reported that HSP 90α is integral to the process of cancer cell invasion. The matrix metalloproteinase (MMP) family plays a central role in cancer invasion due to its ability to degrade the extracellular matrix (Curry and Osteen, 2003). This degradation allows for new blood vessel formation. In addition, the digestion of the matrix enables cells released from the primary tumor mass to migrate to secondary targets (Hanahan and Weinberg, 2000). Although the exact mechanism is unknown, it is now thought that HSP 90 activates MMP-2, which in turn promotes tumor cell invasion. In order for this to occur, the normally intracellular localization of HSPs needs to be considered. Notably, HSP 90 was found outside the cell, where MMPs are activated (Eustace et al., 2004). They also showed that inhibition of extracellular HSP 90 caused an 80% reduction in the active MMP enzyme. This loss of MMP function was directly related to the

loss of invasiveness, which was restored by adding back activated MMPs to the cell system.

Another study example of HSP 90 in the extra cellular environment is in cattle where HSP 90 is secreted into the follicular fluid and is thought to be involved in follicular maturation (Driancourt et al., 1999;Hunt and Morimoto, 1985). These studies clearly indicate HSP 90 is not restricted to the intracellular compartment. At this point, little is known about the mechanism(s) of by which HSP 90 is released outside the cell, nor do we know if other HSPs are similarly secreted.

Heat Shock Proteins and Steroid Receptors

HSP 70 and 90 both have roles in addition to their ability to bind, re-nature and release proteins. When working in concert, HSP 90 and 70 function together to form the machinery required for the ATP – dependent glucocorticoid receptor (GR) (Pratt et al., 2004). HSP 90's involvement in the glucocorticoid receptor has been a subject of study over the last 10 years. A complex consisting of HSP 70, HSP 90 and a small protein, Hop, is formed. Hop does not act as a chaperone but rather binds the two HSPs together. This complex was shown to be sufficient to transform steroid hormone receptors from the nonsteroid binding form to the steroid binding form (Dittmar and Pratt, 1997). This complex was more efficient once the protein p23 was present. Binding of the hormone to the receptor leads to receptor dimerization and appears to decrease its affinity for HSP 90 (Wegele et al., 2004), making it unable to bind with HSP 70 and Hop again.

Similar machinery is required for the progesterone receptor complex. HSP 70, HSP 40, Hop, HSP 90 and p23 are required. Initially the receptor is recognized by HSP 70 and HSP 40. Once bound, HSP 70 ATP is converted to ADP, and Hop-HSP 90 can be recruited to the receptor. After Hop and HSP 70 are released, binding with p23 occurs. At this point, the exact role of p23 is not known but it appears to be required for greater hormone binding (Kosano et al., 1998). After the receptor is activated, progesterone can bind. Once the steroid is bound, the HSP complex is released and the receptor travels to the nucleus where it can activate DNA transcription and the production of a new protein.

Regulation of Heat Shock Proteins

Within seconds of heat shock or other physiological stressors, heat shock transcription factor is activated and begins the rapid translation of genes encoding HSPs (Morimoto, 1993). Heat shock factors (HSF) are remarkably conserved among species ranging from yeast to human. Multiple distinct HSF genes exist, with HSF1, HSF2 and HSF3 found in human (Rabindran et al., 1991), mouse (Sarge et al., 1991), chicken (Nakai et al., 1995) and tomatoes (Scharf et al., 1990). HSF4 is found in mouse (Tanabe et al., 1999) and human (Nakai et al., 1997). All HSFs have two highly conserved features, an amino terminal localized DNA binding domain of approximately 100 amino acids and a motif of hydrophobic heptad repeats (Muller et al., 2004). The terminal end is less conserved and contains the transcription activation domain.

Multiple HSFs indicate that they have different roles in the HSP response. HSF 2 does not have known roles in activation of HSPs through heat or other

environmental stimuli. It is thought that it is activated perhaps during differentiation or development (Morimoto et al., 1992). Little is know about HSF 3 and 4.

Heat Shock Factor 1

HSF1 regulates HSP synthesis and activates HSP gene transcription. HSF1 is the most prevalent HSF and is responsible for activation of HSP genes under thermal and oxidative stress (Wu, 1995). It is one of the more widely studied HSFs. A DNA-binding domain comprising a winged, helix-turn-helix motif is located near the amino terminus. The domain is capable of interacting with the heat shock element (HSE) sequences present in the promoters of HSP genes (Westwood et al., 1991)

Trimerization is necessary for the function of HSF1. In Drosophila, HSF1 is found in the nucleus before and after heat shock (Westwood et al., 1991). In mammalian cells, HSF1 is distributed throughout the cytoplasm in unstressed cells. HSF 1 knockout in mice elicits prenatal death and other developmental defects, indicating that it is necessary for more than just the response to thermal stress (Xiao et al., 1999).

HeLa cells grown at 37°C respond by producing greater levels of HSF 1 when temperatures are increased to 42°C (Morimoto et al., 1996). HSF 1 induction has been shown primarily in response to elevations in temperature, whereas other HSFs such as HSF 2 are activated under different environmental cues, such as exposure to hemin (Sarge et al., 1993).

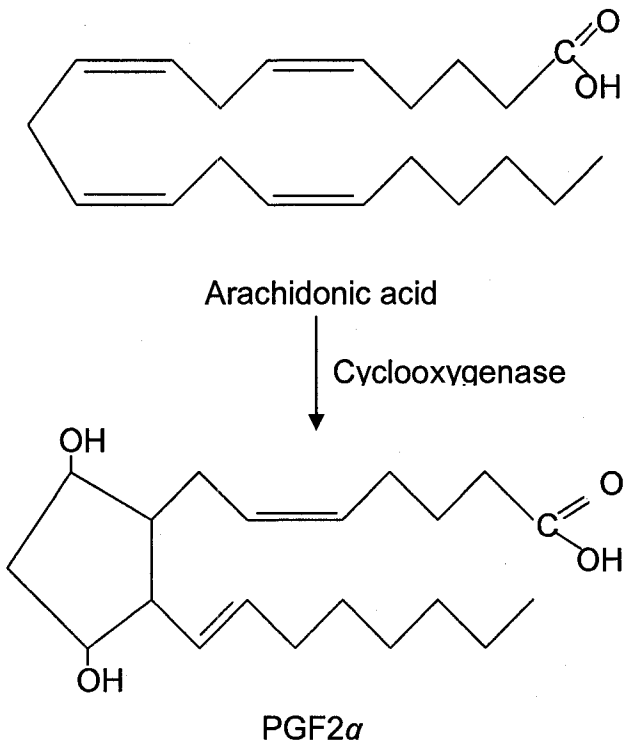
The mechanism involving the activation of HSP 70 requires that there are increased levels of unfolded protein after an initial heat shock. The sequestration of HSP 70 and other chaperones releases HSF 1 from its negatively regulated state and moves it to the nucleus. Once in the nucleus, HSF 1 trimerizes and binds to the HSE in the promoter of HS genes. HSF 1 is then phosphorylated which correlates with elevated transcription of HSP 70 (Morimoto et al., 1992). As HSP 70 helps to refold proteins to their functional tertiary structure, the released HSP 70 re-associates with HSF 1 and triggers the release of more HSF from the HSE (Morimoto et al., 1996).

Prostaglandins

While the body responds to environmental influences by producing heat shock proteins, physiological insults within the body can also provoke a similar response. One such class of molecules which elicits HSP expression is the prostaglandins. Prostaglandins are secreted from cells and act as paracrine/autocrine messengers by diffusing from their source to nearby cells. They promote fever, inflammation and pain, and have many other roles within the body. Prostaglandins are found in almost every tissue of the body and are derived from arachidonic acid (Bergstrom, 1966). The first observation of prostaglandin was made by the gynecologists Kurzrok and Lieb in 1930. They discovered that human semen caused either contraction or relaxation of the human uterus. More than 13 different prostaglandins have since been discovered in human seminal plasma (Hansson and Samuelsson, 1965).

Prostaglandins are eicosanoids and are converted from arachidonic acid to prostaglandin by the enzyme cyclooxygenase. They are lipids consisting of 20-carbon unsaturated hydroxyl fatty acids attached to a five-sided carbon ring. The letter designation of each PG indicates the number of carbon double bond in the side chain.

Figure 3. PGF2α structure



PGF2α

In the rat, HSP 70 induction has been shown to mediate luteal regression (Khanna et al., 1995). In the ovine CL, there was a significant increase in HSP 70 after treatment with PGF2α (McPherson et al., 1993). This signifies that there

is a connection between HSP 70 induction and normal cell death (apoptosis) during PGF2α induced luteolysis.

Another study treated ewes with PGF2α *in vivo* (Murdoch, 1995) and found that an increase in HSP 70 accompanies the increase in apoptosis, or programmed cell death, and the reduction of progesterone production. This suggests a role for HSP 70 in luteolysis but its mechanism of action is open to speculation. Whether HSP 70 induction occurs due to protein degradation associated with PGF2α or stimulates apoptosis is not known.

Objectives

Reproductive failure is an economic concern to dairy farmers. Though much is known about reproduction of bovines, including the detailed structure and function of the CL, it is not known whether the heat shock proteins 70 and 90 react to the stimuli of heat or PGF2α. Therefore, the aim of the present study was to determine (a) the temporal protein expression of HSP 70, HSP 90 and their transcription factor, HSF 1, in bovine luteal cells after heat treatment *in vitro*, and (b) to determine whether PGF2α regulates the temporal protein expression of HSP 70, HSP 90 and HSF 1 *in vitro* and *in vivo*.

CHAPTER II

THE EXPRESSION OF HEAT SHOCK PROTEIN 70, HEAT SHOCK PROTEIN 90 AND HEAT SHOCK FACTOR 1 WITHIN THE MID-CYCLE BOVINE CORPUS LUTEUM AFTER TREATMENT WITH HEAT *IN VITRO* AND PGFα *IN VITRO* AND *IN VIVO*

Introduction

Heat shock proteins (HSP) are ubiquitously expressed in most cell types and are inducible under a variety of environmental and physiological conditions. They are molecular chaperones that safeguard cells against physiological and environmental changes by stabilizing the three-dimensional tertiary structure of proteins and protecting them from degradation (Ellis, 1987). Furthermore, these proteins are important to a cell not just under stressful conditions, but they are also expressed constitutively to maintain protein-protein interactions (Lindquist, 1986). The genes encoding heat shock proteins are highly conserved throughout evolution with representatives from distant prokaryotic and eukaryotic species having at least 50% identical coding (Hunt and Morimoto, 1985). Even thermophilic organisms, whose optimum growth temperature ranges between 50-

90°C respond to a sudden temperature rise by rapidly increasing expression of HSP (Neuer et al., 2000).

The members of the HSP family are named by their molecular weight and not by their structure or function (Csermely et al., 1998). They are subdivided into small HSPs, such as HSP 25 and 27, and large HSPs, such as HSP 70 and 90. While different stimuli elicit an induction of HSPs, the cells response is usually mediated by the expression of transcription factors, heat shock factor (HSF) 1 and 2. It seems, though, that the nature of response is dependent on the type of stimulus. For example, increases in temperature signal HSF 1 expression, while other chemical signals like ischemia and heavy metals induce the expression of HSF 2 (Trinklein et al., 2004).

The heat shock proteins 70 and 90 are of the most interest in the present study. Heat shock protein 70 is well known for its ability to assist folding of proteins (Bukau et al., 2000;Mayer, 2005;Zhang et al., 2002) by preventing aggregation (Wegele et al., 2004) of hydrophobic surfaces so that unique tertiary structures are formed. In addition, HSP 70 is an important component of the steroid hormone receptor complex (Pratt et al., 2004). Interestingly, growing oocytes express high levels of HSP 70 but fully developed oocytes are unable to express HSP 70. This could possibly explain why mammalian oocytes have an increased sensitivity to heat stress (Neuer et al., 2000). In the rat (Pratt and Toft, 2003) and sheep (McPherson et al., 1993), HSP 70 expression increases following the administration of PGF2α. It is thought that HSP 70 induction

mediates luteal regression in rats (McPherson et al., 1993;Khanna et al., 1995) by severely impairing steroidogenesis.

Heat shock protein 90 is abundant in most if not all unstressed prokaryotic and eukaryotic cells (Kochevar et al., 1991;McPherson et al., 1993), accounting for 1-2% of cytosolic proteins (Pratt, 1997). Nearly 100 proteins are known to be regulated by HSP 90 (Pratt and Toft, 2003). Among its many functions, HSP 90 is a "cradle-to-grave" chaperone for steroid receptors (Pratt, 1997), regulating proper receptor folding, trafficking, transcriptional activation and turnover. HSP 90 is also expressed in somatic cells of the bovine ovarian follicle. It is released into the follicular fluid (Driancourt et al., 1999), and may have a role in follicular maturation.

While it is well documented that heat stress decreases fertility in dairy cows (West, 2003), it is not known whether HSPs are involved. Furthermore, knowledge is lacking regarding the relationship between PGF2α and HSP expression in the bovine CL. Therefore the aims of the present study were (a) to determine the temporal protein expression of HSP 70, HSP 90 and HSF 1 in bovine luteal cells after heat treatment *in vitro*, and (b) to determine whether PGF2α regulates the temporal protein expression of HSP 70, HSP 90 and HSF 1 *in vitro* and *in vivo*.

Materials and Methods

Animals

Corpora lutea for the *in vitro* and *in vivo* experiments were collected from mid-cycle dairy cows by colpotomy (Towle et al., 2002). Cows were housed at the University of New Hampshire (UNH) Fairchild Dairy Teaching and Research Center and (enter West Virginia info here). Approvals were received from The Institutional Animal Care and Use Committees (IACUC) at UNH (051103).

Luteal Cell Culture

Under aseptic conditions, the CL was weighed, minced into approximately 2 mm^3 pieces and dissociated with collagenase (type 1, 2000 U/g tissue; Worthington Biomedical, Freehold, NJ) in phenol red-free Ham's F-12 medium (Gibco Life Technologies, Grand Island, NY) containing gentamicin (30 mg/ml; Gibco Life Technologies, Grand Island, NY) and 0.5% bovine serum albumin (BSA; Sigma, St. Louis MO). Briefly, two one-hour dissociations at 37°C were performed. The combined cell suspension was subjected to three sequential centrifugations (150, 75, 57 ×g), each for 10 minutes. Cell viability was determined by trypan blue dye exclusion and cell number was determined with an ocular micrometer. Culture flasks (25 cm^2; Corning Inc., Corning NY) were coated with Ham's F-12 containing 10% fetal bovine serum, which was removed after one hour. Then, flasks containing 4 ml of Ham's F-12 medium containing gentamicin (30 mg/ml; Gibco Life Technologies, Grand Island, NY) supplemented

with insulin (5 µg/ml), selenium (5 ng/ml) and transferrin (5 µg/ml) (Upstate Biotechnology, Lake Placid, NY) were seeded with $1x10^6$ viable luteal cells/ml and incubated in a closed system for 24 hours at 37°C to allow cell attachment. Afterwards, spent medium was replaced and cells were either subjected to heat or treated with PGF2α. At the end of each time point, conditioned medium was collected and stored at -20°C until analysis. The cells were prepared for protein extraction.

Protein Extraction and Determination of Protein Concentration

The cells from triplicate flasks for each treatment were combined for protein extraction using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 10 mM EDTA, 1% [v/v] Triton X-100, pH 7.4) (Zhang et al., 2002) with protease inhibitors (5 µg/ml AEBSF, 2 µg/ml pepstatin A, 10 µg/ml aprotinin). After a freeze-thaw cycle, the cell extracts were sonicated (Sonifier Cell Disruptor 350, Branson Sonic Power Co.) to disrupt cell membranes and release proteins. Following centrifugation for 10 minutes at 10,000 x g, the supernatant was frozen at -20°C until analysis.

Luteal tissue was extracted with lysis buffer containing protease inhibitors (1 µg/ml AEBSF, 1 µg/ml pepstatin A, 10 µg/ml aprotinin) in a ratio of 1g tissue: 8 ml lysis buffer. Tissue was minced into 2 mm^3 pieces and homogenized (two pulses) with a Brinkmann Kinematica Polytron, as previously described (Towle et al., 2002). The resulting homogenate was then sequentially centrifuged at 800 x g and 10,000 x g, each for 10 mins at 4°C. Supernatants were then sonicated and stored at -20°C until analysis.

Immunoblotting

HSP 70, HSP 90, and HSF1 proteins were detected using a previously described immunoblotting protocol (appendix B). Samples were reduced (with the exception of HSF1) at 100°C for 3 minutes with 2-mercaptoethanol (4%; Sigma, St. Louis, MO). Equivalent luteal protein extracts were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) and then electrophoretically transferred onto nitrocellulose membranes (0.45 um; Schleicher & Schell, Keene NH). The membranes were then incubated at room temperature with 5% non-fat dry milk in TBST (0.010 M Tris, 0.15 M NaCl, 0.04% Tween 20). Respective primary antibodies (4 µg/ml Mouse Anti-HSP 70 Monoclonal Antibody, Sigma-Aldrich, Saint Louis, MO; 4 µg/ml Mouse Anti-HSP 90 Monoclonal Antibody, Assay Designs, Inc. Ann Arbor, MI; 2 µg/ml Rat anti-HSF 1, Assay Designs, Inc. Ann Arbor, MI) were added and incubated overnight at 4°C. A series of washes with TBST (4 times each for 10 min) followed, before the addition of the secondary antibody (all at 1:10,000; anti-rat IgG for HSF 1 and anti-mouse IgG for HSP 70 and HSP 90), with each conjugated to horseradish peroxidase (Pierce, Rockford, IL), and incubated at room temperature for 1 hr. Membranes were then washed again with TBST (4 times each for 10 min) before chemiluminescent substrate (Super Signal West Pico Substrate; Pierce, Rockford; IL) was added for visualization. This was followed by exposure to photographic film (Kodak X-Omat) and processing in a Konica (Wayne, NJ) automatic developer. Molecular weight markers (Precision Plus Protein Dual

Color Standards; Biorad Hercules, CA) were run in an adjacent lane. A HeLa cell lysate (Assay Designs, Inc. Ann Arbor, MI) served as a positive control for HSP 70 and HSF-1 while a HSP 90 recombinant protein (Assay Designs, Inc. Ann Arbor, MI) served as the positive control for Hsp90. Negative controls include incubation with mouse and rat normal serum in place of primary antibody, and incubations excluding the respective primary antibody. Beta actin (1:1000 Sigma-Aldrich Co, St. Louis, MO) was used to confirm equivalent protein loading in all lanes (appendix D).

Radioimmunoassay

The progesterone (P_4) content in conditioned medium was determined by radioimmunoassay (RIA). The methods and parameters are described (appendix C). The antibody was obtained from Gordon Niswender and used at a final dilution of 1:10,000. Tritiated P_4 (1,2-^{3}H; 53 Ci/mmol) was obtained from New England Nuclear (Boston, MA). After charcoal adsorption and centrifugation, the supernatant was decanted into mini-vials, and Ready Safe Cocktail (Beckman, Columbia, MD) was added. Radioactivity was determined in a liquid scintillation counter (Beckman LS 6000 IC, Palo Alto, CA). The intraassay coefficient of variation was 8.7%, and the interassay coefficient of variation was 15.5% .

General Procedures

Experiment 1

The objective was to determine the effects of heat on protein expression of HSP 70, HSP 90, HSF 1 and progesterone production by mid-cycle bovine luteal cells *in vitro*. Mid-cycle (day 10 or 11) bovine corpora lutea (n = 4) were dissociated as described above. Cells were placed in T-25 flasks and randomly divided into treatment groups (3 replicate flasks per treatment). Luteal cells were incubated at 42°C for 10 min, 2 hr or 24 hr for each time point, and contemporaneous controls were incubated at 37°C. Following each treatment period, all flasks were placed in a 37°C incubator for 2 hr (recovery period) before conditioned medium was collected. Protein extraction from cells was performed as described above before immunoblotting. Additional flasks were also set up at the 24 hr time-point which enabled a determination of luteal cell viability using trypan blue.

Experiment 2

The objective was to determine the effects of PGF2α on protein expression of HSP 70, HSP 90 and HSF 1 by mid-cycle bovine luteal cells *in vitro*. Mid-cycle (day 10 or 11) bovine CL (n = 4) were dissociated as described above. Luteal cells were placed in T-25 flasks and randomly divided into treatment groups (3 replicate flasks per treatment), which received either 0, 10, 50, 100 or 500 µg/ml of PGF2*α*. Flasks were incubated at 37° C for 0.5 or 24 hr. Afterwards,

conditioned medium was collected and protein extraction was performed as described above, before immunoblotting.

Experiment 3

The objective was to determine the effects of PGF2α on protein expression of HSP 70, HSP 90 and HSF 1 by bovine CL *in vivo*. Mid-cycle (day 10 or 11) cows received either saline (n = 4) or a luteolytic dose (15mg) of PGF2α (intramuscular; Lutalyse), and CL were collected 30 min (n = 4) and 24 hr (n = 4) post injection. Luteal tissues were stored in a -80°C freezer until protein extraction, as described above, before immunoblotting.

Statistical Analysis

The HSP 70, HSP 90 and HSF 1 protein bands and their respective HeLa cell lysate/recombinant proteins were densitized using UN-SCAN-IT version 6.1 (Silk Scientific Ind., Orem, UT). Each CL sample was run in duplicate. The pixel values for HSP 70, HSP 90 and HSF 1 in all CL samples for a particular time-point or treatment were averaged and then normalized against the respective positive controls (i.e. HeLa cell lysate or recombinant protein). The resulting ratios were then analyzed by one-way analysis of variance (ANOVA) (SYSTAT 10, Inc., Evanston, IL), followed by a Tukey's test to determine the effects of treatment or time. Differences in P_4 concentrations among treatments or time were determined by one-way ANOVA (SYSTAT 10, Inc., Evanston, IL) followed by a Tukey's test.

Results

Experiment 1: Effect of heat on protein expression of HSP 70, HSP 90, HSF 1 and progesterone production by mid-cycle bovine luteal cells in vitro

Visual observations of immunoblots revealed a protein with a relative molecular mass (M_r) of approximately 72kDa, which co-migrated with the HSP 70 protein in the HeLa cell lysate (FIG 4A). Exclusion of the primary antibody, and substitution with normal mouse serum (HSP 70 and HSP 90) or normal rabbit serum (HSF 1) served as negative controls, which resulted in the absence of specific bands (data not shown). Densitometric analysis revealed that the expression of HSP 70 in heat treated cells at 24 hr was greater ($p<0.05$) than the controls at the 10 min, 2 hr and 24 hr time points and the heat treated cells at 10 min, but not ($p>0.05$) the heat treated cells at 2 hr.

In luteal cell extracts, a protein with an M_r of approximately 94kDa was present, which co-migrated with the recombinant HSP 90 protein (FIG 4B). Similar to HSP 70, densitometric analysis revealed that HSP 90 expression in cells heated at 42°C for 24 hrs was greater ($p<0.05$) than the controls at the 10 min, 2 hr and 24 hr time points and the heat treated cells at 10 min, and 2 hr.

In order to determine a possible mechanism by which heat treatment induces HSP expression, the transcription factor HSF1 was studied. Although a protein with an M_r of approximately 95kDa was present in luteal cell extracts and

co-migrated with the protein in our positive control, densitometric analysis revealed no change ($p>0.05$) in HSF 1 protein expression at any time point in control or heat treated cells (FIG 4C).

To assess the effects of heat at 42°C on luteal cell function, progesterone radioimmunoassay was performed on the conditioned medium. The only change observed was at the 24 hr time point, when progesterone concentration was greater ($p<0.05$) in the heat treatment than the respective control (FIG 5). In addition, when 24 hr heat treated cells were stained with trypan blue, 88.3% of them were viable (data not shown).

Experiment 2: Effect of PGF2α on protein expression of HSP 70, HSP 90 and HSF 1 by mid-cycle bovine luteal cells in vitro.

Our goal was to determine whether PGF2α induces the expression of HSP 70, HSP 90 and HSF 1 by bovine luteal cells in a similar fashion as heat. Immunoblots revealed a protein with an M_r of approximately 72 kDa, which co-migrated with HSP 70 in the HeLa cell lysate and was present in all samples (FIG 6A and 6B). However, densitometric analysis revealed that HSP 70 expression in cells treated for 0.5 hr and 24 hr in the absence (control) or presence (10, 50, 100 or 500 µg/ml) of PGF2α did not change ($p>0.05$) (FIG 6A and 6B).

Similarly, while HSP 90 (FIG 6C and 6D) and HSF 1 (FIG 6E and 6F) were expressed by luteal cells, densitometric analysis revealed that neither protein changed ($p>0.05$) due to time or PGF2α concentration.

Experiment 3: Effects of PGF2α on protein expression of HSP 70, HSP 90 and HSF 1 by bovine CL in vivo.

Our goal was to determine the in vivo expression of HSP 70, HSP 90 and HSF 1 following PGF2α treatment. As observed in Experiments 1 and 2, an approximately 72 kDa protein that co-migrated with the HSP 70 in the HeLa cell lysate was present in luteal tissue. Densitometric analysis revealed that there was no difference ($p>0.05$) in the expression of HSP 70 between the saline control, 0.5 hr or 24 hr time points (FIG 7A). In addition, an approximately 94 kDa protein which co-migrated with the HSP 90 recombinant protein was also observed. Unlike HSP 70, densitometric analysis revealed that HSP 90 was greater ($p<0.06$) at the 0.5 hr time-point than the saline control and the 24 hr time point (FIG 7B).

Similar to experiments 1 and 2, the transcription factor, HSF 1, co-migrated with HeLa cell lysate at an M_r of 95 kDa and showed no difference between the saline control and the 0.5 hr and 24 hr time points (FIG 7C).

Figure Legends

Figure 4. Experiment 1. Effect of temperature on protein expression of A) HSP 70, B) HSP 90 and C) HSF 1. Densitometric ratio is expressed as the sample average pixel value normalized against the average pixel value of the recombinant protein or HeLa cell lysate. The data are presented as means ± SEM with dissimilar letters denoting differences ($p<0.05$) between control (■) and heat (□) treated cells. Representative immunoblots are shown for each protein where 1 = 10 min control, 2 = 10 min heat treated, 3 = 2 hr control, 4 = 2 hr heat treated, 5 = 24 hr control and 6 = 24 hr heat treated. Recombinant protein or HeLa cell lysate is denoted with the (+) symbol.

Figure 5. Progesterone production expressed as ng/500,000 cells in control (C24) and heat treated (H24) luteal cells at the 24 hr time point. Data are presented as means ± SEM. Asterisk denotes significance at $p<0.05$.

Figure 6. Experiment 2. Effect of PGF2α concentration and time of incubation on HSP 70 (A, 0.5 hr, B, 24 hr), HSP 90 (C, 0.5 hr, D, 24 hr) and HSF 1 (E, 0.5 hr, F, 24 hr) expression. Data for the 0.5 hr time point are shown on the left; data for the 24 hr time point are shown on the right. Densitometric ratio is expressed as the sample average pixel value normalized against the average pixel value of the recombinant protein or HeLa cell lysate. The data are presented as means ± SEM. Representative immunoblots are shown for each protein for both

timepoints and at each PGF2α concentration (1 = 0, 2 = 10, 3 = 50, 4 = 100, 5 = 500 µg/ml). Recombinant protein or HeLa cell lysate is denoted with the (+) symbol. No differences were observed ($p>0.05$).

Figure 7. Experiment 3. Expression of HSP 70 (A), HSP 90 (B) and HSF 1 (C) at 0.5 hr and 24 hr following PGF2α *in vivo*. Densitometric ratio is expressed as the sample average pixel value normalized against the average pixel value of the recombinant protein or HeLa cell lysate. The data are presented as means ± SEM. Representative immunoblots are shown for each protein and time point (1 = 0 hr, 2 = 0 hr, 3 = 0.5 hr, 4 = 0.5 hr, 5 = 24 hr, 6 = 24 hr). Recombinant protein or HeLa cell lysate is denoted with the (+) symbol. Asterisks denote difference ($p<0.05$) between treatments.

Figure 4 Experiment 1. Effect of temperature on protein expression of HSP 70, HSP 90 and HSF 1

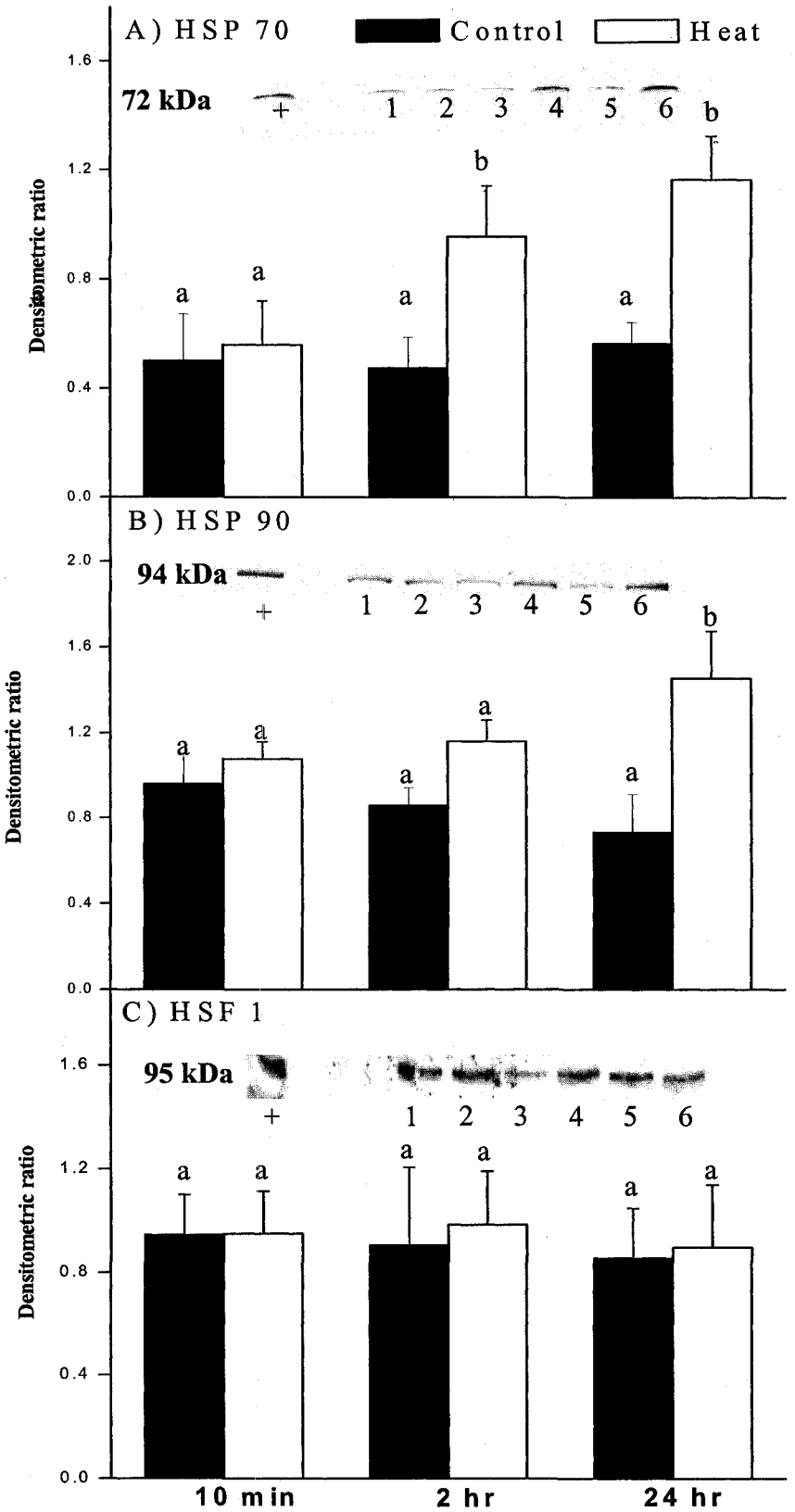


Figure 5. Progesterone production expressed as ng/500,000 cells in control (C24) and heat treated (H24) luteal cells at the 24 hr time point.

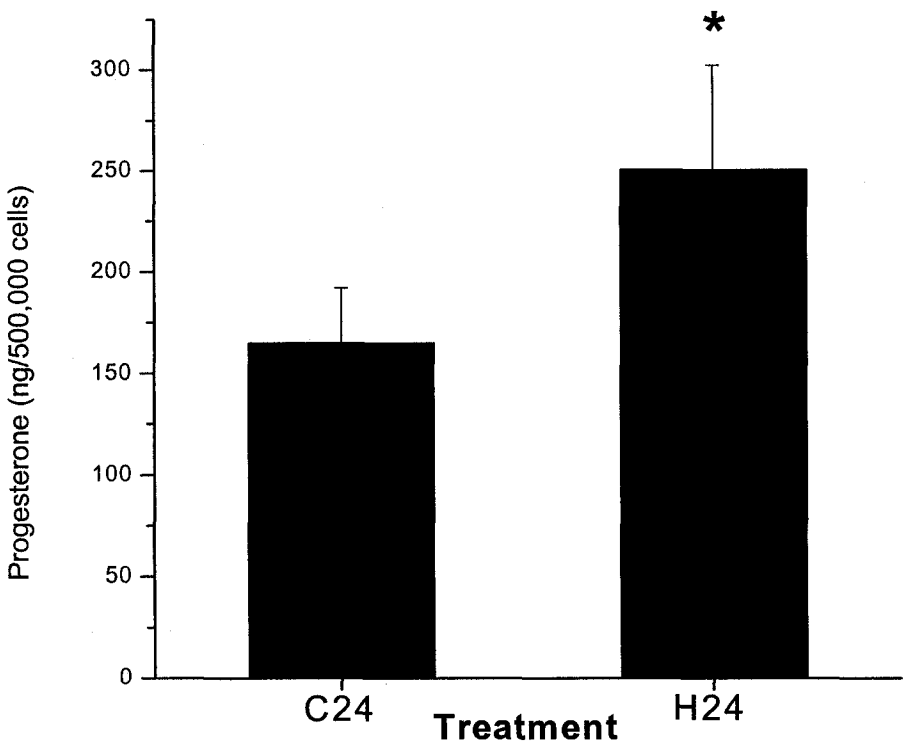


Figure 6. Effect of PGF2α concentration and time on HSP 70, HSP 90 and HSF 1 expression.

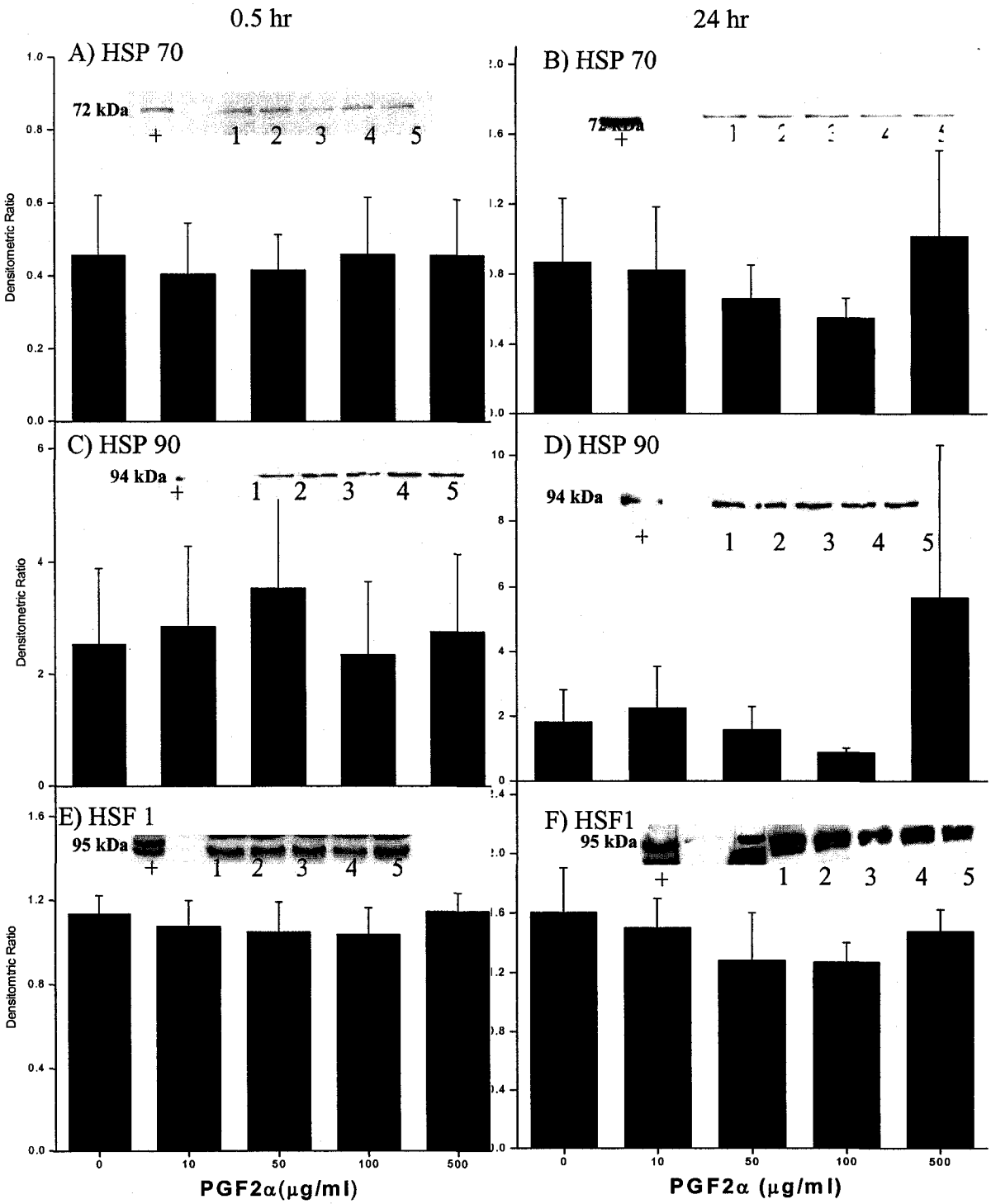
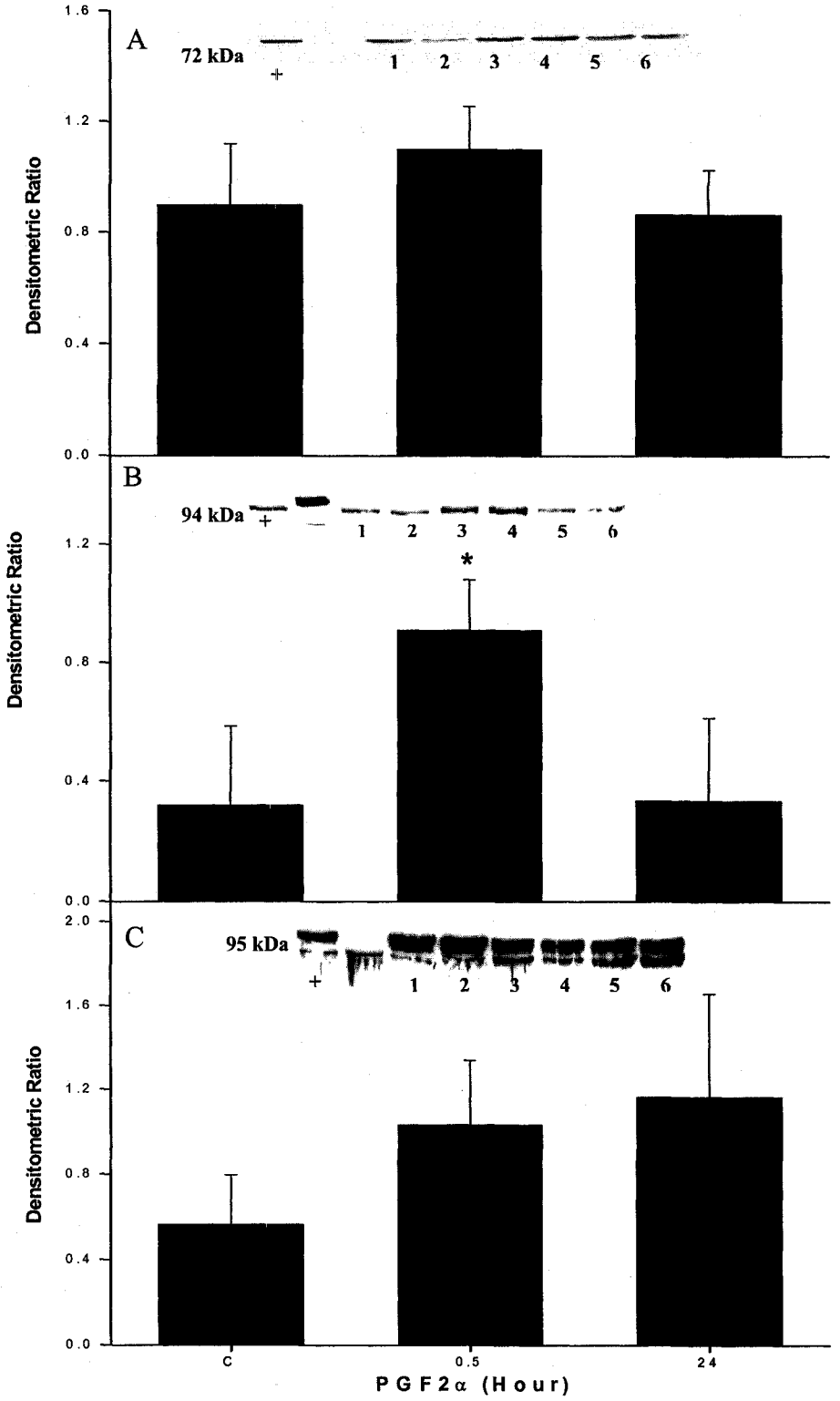


Figure 7. Expression of HSP 70, HSP 90 and HSF 1 at 0.5 hr and 24 hr following PGF2*α in vivo*.



Discussion

The bovine corpus luteum receives a multitude of stimuli, both from the external environment, such as heat, and from the internal environment, such as PGF2α. In turn luteal cells react by upregulating HSPs, in part, to protect the functional structure of its proteins. In the present study, we found that HSP 70 and HSP 90 protein expression was increased after heat treatment (HSP 70 at 2 hr and 24 hr, HSP 90 at 24 hr) *in vitro* and HSP 90 protein expression was increased after PGF2α treatment at 0.5 hr *in vivo*. While it is difficult to compare *in vivo* and *in vitro* results, luteal cells may respond to heat and PGF2α treatment differently.

To our knowledge, this is the first report of HSP 90 protein expression by the bovine CL after PGF2α treatment *in vivo*. Previous studies in the ewe showed that there was an acute increase in HSP 70 expression after administration of PGF2α *in vivo* (Murdoch, 1995). In contrast, neither HSP 70 nor HSP 90 protein expression was influenced by PGF2α treatment *in vitro*. While this differed from the response of rat luteal cells (Khanna et al., 1995) where HSP 70 expression was increased, the lack of response by bovine luteal cells, however, may not be altogether surprising. It is well known that the bovine luteal cell, *in vitro*, responds to PGF2α by increasing (Hansel, 1973;Hixon and Hansel, 1979) or decreasing (Benhaim et al., 1987) progesterone production. Such a differential response may be due to the loss of cell to cell communication

(Miyamoto et al., 1993) in an *in vitro* system, or the absence of other cell types or byproducts typically found *in vivo*. For example, Murdock (Murdoch, 1995) suggests that *in vitro* culture of luteal cells may lack the immune-inflammatory inputs that are present in *in vivo* systems. Further, HSP 70 is known to function in major histocompatibility (MHC) processing and presentation as well as being a possible target for immune recognition (De Nagel and Pierce, 1991).

The more robust expression of HSPs by bovine luteal cells in response to heat may be attributed to an evolutionarily conserved pathway that is ubiquitous among living organisms. Heat shock proteins are present in the most primitive eukaryotes, bacteria and plants (Sarge and Morimoto, 1991). In addition, heat stress produces chromosomal puffing in Drosophila DNA (Ritossa and Vonborstel, 1964), and it is known that even thermophilic organisms, whose optimum growth temperature ranges between 50-90°C, respond to a sudden temperature rise by rapidly increasing expression of HSP (Neuer et al., 2000). Thus, it is possible that this primitive reaction to heat is readily manifested by cells *in vitro*, while the pathways associated with PGF2α treatment require the key players (e.g. the immune-inflammatory factors) that are normally present *in vivo*, but are absent *in vitro*.

Alternatively, differences in HSP expression between heat and PGF2α treatments may be due to intrinsic signal transduction pathways elicited by these stimuli. The most likely candidates are the transcription factors, HSF 1 and HSF 2. Under the conditions of the present experiment, no differences were observed in any of the studies. Although the possibility exists that other transcription

factors may work in the CL, a more likely explanation is the rapidity of HSF 1 expression. HSF 1 protein expression increases within seconds of temperature elevation, which results in a rapid increase in the transcription of genes encoding HSPs (Morimoto et al., 1996). In the system used in the present experiments, even the 10 min time-point may have been too long for us to detect a change in HSF 1 protein expression. In future studies, perhaps determining mRNA expression will provide insight towards the relationship between HSF and other treatments like heat and PGF2α.

The hallmark of CL function is progesterone biosynthesis (Short, 1962). It is known that heat, *in vivo*, decreases progesterone concentration. Howell (1994) conducted a study in which Holstein cows were subjected to long term hot summer temperatures (Howell et al., 1994). This study showed that the lower progesterone concentrations were not due to decreased CL size or a shorter luteal phase, but rather it was due to decreased luteal cell function. Wolfenson (2000) performed *in vitro* studies comparing luteal cells from CLs obtained during the summer and winter, and showed that CLs obtained during the summer had decreased levels of progesterone.

The data from these studies contrast with the increased progesterone ($p<0.05$) concentration found in the present study. However, other studies have also reported increased progesterone concentrations after heat treatment (Abilay et al., 1975;Trout et al., 1998;Wilson et al., 1998b;Trout et al., 1998). The increased progesterone concentration is thought to be due to decreased estradiol synthesis, resulting in a longer luteal phase during the estrous cycle (Wilson et

al., 1998b). The differences in progesterone production could also be attributed to the type of heat treatment. Cows exposed to chronic high temperatures, such as in the tropics, often experience decreased progesterone concentrations while cows exposed to acute temperature increases, such as in a heat chamber, experience higher concentrations of progesterone (Howell et al., 1994). Because the luteal cells in the current study experienced an acute high temperature, the increase in progesterone concentration is not altogether surprising although the exact mechanism is not known at this time.

Similar to heat treatment, the response to PGF2α by luteal cells is also variable. Many studies have documented the ability of PGF2α to decrease progesterone production by luteal cells. For example, Hall et al., (1979) and O'Grady et al., (1972) documented inhibition of basal progesterone production in the rat after treatment with PGF2α *in vitro*. On the other hand, other studies found that PGF2α increases progesterone production by bovine luteal slices (Hansel, 1973) and bovine luteal cells *in vitro* (Hixon and Hansel, 1979). However, the data from the current study showed no changes in progesterone concentration after exposure to PGF2α at any concentration or time point. It is quite possible that the time of exposure to PGF2α is important. Another study found that more than 24 hr are required to elicit a response to PGF2α by bovine luteal cells *in vitro* (Pate and Condon, 1989). The longest incubation period in the current study was 24 hr, suggesting that this may not be enough time to show a response.

How external environmental conditions, such as heat, and internal (physiological) stimuli, such as PGF2α affect luteal cells are not completely known. The present study showed that heat and PGF2α increased the expression of HSPs in the bovine CL. It would appear that these treatments utilized pathways other than HSF 1 to elicit their responses by the CL. The response to heat is likely to have evolved from the first primitive organisms to mammals; and the HSPs expressed in the cow CL are thought to have at least 50% identity (Hunt and Morimoto, 1985) to distant prokaryotic and eukaryotic species. On the other hand, the response to PGF2α may involve other pathways that are not present in our *in vitro* system including immune interactions and cell to cell contact. Future studies are needed to provide insight.

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APPENDACIES

APPENDIX A. PROTEIN CONCENTRATION DETERMINATION (BRADFORD ASSAY)

Part 1

1. Turn on spectrophotometer and allow to warm up before using.

2. Make up standards tubes

Tube #	BSA	PBS	Reagent*
1	0	800µl	200
2	0	800µl	200
3	4µl (2ug)	796µl	200
4	4µl (2ug)	796µl	200
5	8µl (4ug)	792µl	200
6	8µl (4ug)	792µl	200
7	12µl (6ug)	788µl	200
8	12µl (6ug)	788µl	200
9	16µl (8ug)	784µl	200
10	16µl (8ug)	784µl	200
11	20µl (10ug)	780µl	200
12	20µl (10ug)	780µl	200

*Add reagent as last step when all other sample tubes are made up.

3. Prepare sample (unknown tubes) depending on concentration of sample it may be necessary to make a dilution (example 1:10)

4. Make duplicate tubes and plan to have two different aliquot sizes for each sample to total 4 tubes for each sample. Add appropriate amount of PBS so each tube totals 800µl before the addition of the reagent.

5. Add Bradford reagent, 200µl to each tube

6. Vortex and set aside to incubate at room temperature for 15 minutes

7. Read at OD 595 and record readings.

APPENDIX B. IMMUNO (WESTERN) BLOTTING

SOLUTIONS:

Ammonium Persulfate Solution
(APS; 100mg/ml
20 mg ammonium persulfate
200 ml ddH_2O

1.5M Tris (pH 8.8)
18.17g Tris
100 ml ddH_2O

0.5M Tris (pH 6.8)
6.06g Tris
100 ml ddH_2O

Sample Buffer (10 ml)
10 ml 0.25 M Tris (pH 6.8)
1 g SDS
400 mg Sucrose
10 mg Bromophenol Blue

0.25M Tris (pH 6.8)
3.03g Tris
100 ml ddH_2O

Separation Gel (12% acrylamide)
6.525 ml ddH_2O
3.75 ml 1.5 M Tris (pH 8.8)
4.5 ml 40% acrylamide
150 µl 10% SDS
75 µl APS (100mg/ml)
7.5 µl TEMED

Stacking Gel (4% acrylamide)
6.36 ml ddH_2O
2.52 ml 0.5 M Tris (pH 6.8)
1 ml 40% acrylamide
100 µl 10% SDS
50 µl APS (100 mg/ml)
10 µl TEMED

Electrode Buffer 10x
30.0 g Tris
144.0g Glycine
5.0g SDS
Bring up to 1L with ddH_2O

Blotting Buffer
800 mL ddH_2O
200 mL Methanol
15.03 g Glycine
3.15g Tris

5% Milk
2.5 g evaporated non-fat powdered milk
50 ml TBST buffer

TBST Buffer
1.2 g Tris
8.8 g NaCl
0.5 ml Tween 20
QS to 1L

PROCEDURE:

Part 1. Casting gels and electrophoresis

1. Set up gel apparatus:
    a. Put large and small glass plates together separated by two black spacers, one on each side
    b. Slide into casting component making sure both plates and spacers are even with the bottom.
    c. Tighten knobs
    d. Cover bottom with parafilm and snap into tower with tightening knobs facing away from you.
    e. Repeat with the other side, snapping second glass assembly into the opposite position on the snap tower.

2. Casting the separation gel:
    a. Add ddH_2O, 1.5M Tris, 40% acrylamide (wear gloves), 10% SDS and APS
    b. Add TEMED when ready to load into casting assembly. Have a glass Pasteur pipette ready with bulb on the top.
    c. After adding TEMED swirl to mix and quickly load gel solution between the two glass plates of the apparatus assembly, fill to notch present on snap tower
    d. With a clean pipette slowly add ddH_2O on top of the gel solution
    e. Allow to polymerize for 30-45 min.

3. Casting stacking gel:
    a. Add ddH_2O, 0.5M Tris, 40% acrylamide (wear gloves), 10% SDS and APS.
    b. Pour the ddH_2O off from the casting assembly and blot dry with a kimwipe.
    c. Add TEMED, swirl to mix and pipette on top of separation gel.
    d. Immediately insert 1mm thick 10 well comb. Center side to side
    e. Allow to polymerize, about 30 min.

4. Preparation of standards and samples:
    a. While stacking gel is polymerizing, make up samples and standards for a total volume of 30 µl in each tube. 15µl should be sample and appropriate volume of CAB, the other 15µl should be sample buffer

5. Loading samples:
    a. When stacking gel has polymerized, carefully pull out comb.
    b. Remove gels/glass assemblies from snap tower and insert into electrode tower.
    c. Place new assembly into plastic container and add ~800 ml 1x electrode buffer. Pour directly into wells to fill them

d. Begin loading wells with special loading tips

6. Running the gels:
    a. Once samples are loaded attach electrode (green) cover in proper orientation.
    b. Plug into power source.
    c. Set volts to 200
    d. Start, small bubbles should appear around the gel apparatus
    e. Run until the dye front reaches the bottom, ~30-45min.

Part 2. Electroblotting

1. Preparation for blotting:
    a. Cut two pieces of filter paper and one nitrocellulose membrane in half
    b. When gels are almost finished running, make blotting buffer
    c. Put four fiber pads, filter paper and membranes in a plastic bucket and cover with blotting buffer.
    d. Fit electrode cassette into another plastic bucket with small stir bar and ice pack; fill partially full with blotting buffer.
    e. Take gel assembly out of cassette and carefully separate glass plates. With a spatula cut the top stacking gel off the gel.
    f. Follow diagram shown below to layer fiber pad, filter paper, gel, membrane, filter paper and finally fiber pad all inside a plastic sandwich.
    g. Make sure to remove any air bubbles between the gel and the membrane with a Pasteur pipette. Keep entire assembly moist with blotting buffer and put entire sandwich into the electrode cassette with the black part of the sandwich towards the black colored part of the cassette
    h. Complete the second sandwich in the same way.
    i. Fill plastic bucket with electrode cassette up with the rest of the blotting buffer.
    j. Place electrode cover on tank
    k. Put plastic bucket into a plastic tub and fill with ice.

2. Electroblotting
    a. Plug assembly into power source EC650 and run at a constant 200 milliamps for 2 hours.

Part 3. Membrane treatments

1. Blocking
    a. After electroblotting is complete, remove membranes and place each in their own small tray with 25 ml 5% milk in each tray.
    b. Place trays on shaker set at 2 and shake for 1 hour.

2. Adding the primary antibody.
    a. Primary antibody should be made up in 5% milk and at a dilution of 1:10,000 (read antibody specifications sheet to check your specific antibody's dilution range)
    b. When blocking is complete discard milk.
    c. Pour antibody on top of membrane and cover with parafilm
    d. Make sure there are no air bubbles trapped between membrane and parafilm
    e. Let incubate overnight at 4°C on a rotator.

3. Washing membrane
    a. Remove primary antibody into a 10 ml centrifuge tube and put in freezer to be used again.
    b. Do one quick rinse with TBST, then cover with additional TBST and do 4 sets of washes at 10 minutes each.

4. Secondary antibody
    a. Make up another 50 ml of 5% milk. Add 5µl of secondary antibody. Divide this between the two membranes

5. Washing
    a. Complete a second round of washes, just like the first, 4 sets at 10 minutes each
    b. Before the last wash, turn on the developer

6. Chemiluminescent reagent
    a. Make up West Pico Super Signal, 5 mls of each solution into a 10 ml centrifuge tube. Do not expose mixed solution to direct light
    b. Add chemiluminescents to each membrane, 5 ml each.
    c. Cover with parafilm and put in a dark area for 5 minutes.

7. Set up for darkroom
    a. Place a piece of saran wrap on bench top
    b. Blots excess chemiluminescent reagent from membranes with kimwipe.
    c. Place upside down on saran wrap, and the other membrane below
    d. Cover with filter paper and wrap excess saran wrap around the back of it.
    e. Put in film cassette box and tape in place.

8. In the darkroom
    a. Expose film to membrane from 1-10 minutes (more or less depending on signal strength)

b. Put film in developer
c. Processing takes about 3 minutes.

APPENDIX C: RADIOIMMUNOASSAY OF PROGESTERONE

SOLUTIONS:

PBSG
Gelatin 1.0g
$NaH_2PO_4 \cdot H_20$ 5.38g
$NaH_2PO_4 \cdot 7H_20$ 16.35g
NaCl 9.0g
Na Azide 20% 1.0g
q.s. to 1000ml
pH should equal 7.0

Charcoal Suspension
20 mg Dextran T-70
200mg prewashed Norit A neutral charcoal
100 ml PBSG assay buffer

Progesterone Radioimmunoassay

	Day 1					Day 2
Tube #	Std./sample	(µl)	3H(µl)	AB(µl)	PBSG(µl)	charcoal (µl)
1-3	Total		100	0	300	0
4-6	NSB		100	0	300	750
7-9	"zero"		100	100	200	750
10-12	0.125 ng/ml	100	100	100	100	750
13-15	0.25 ng/ml	100	100	100	100	750
16-18	0.50 ng/ml	100	100	100	100	750
19-21	1.00 ng/ml	100	100	100	100	750
22-24	2.00 ng/ml	100	100	100	100	750
25-27	4.00 ng/ml	100	100	100	100	750
28-30	8.00 ng/ml	100	100	100	100	750
31-33	16.00 ng/ml	100	100	100	100	750
34-36	samples (1:10)	50	100	100	150	750
36-	samples (1:10)	100	100	100	100	750

Procedure
Day 1

1. Aliquot out PBSG, std/sample and AB (1:) dilution. Finally add 3H, using careful technique

2. Cover with parafilm, label with radioactive tape incubate at 4°C overnight

Day 2

1. Turn on centrifuge to 4°C

2. Start stirring charcoal suspension on stir plate on ice

3. Take assay out of fridge and put on ice

4. Add charcoal suspension to all tubes except 1-3, add 750µl PBSG to those, start timer for 10 mins. Place tubes in chilled centrifuge buckets, put in centrifuge and start spinning once timer has gone off

5. Spin at 2200 g for 10 min

6. Pour off supernatant into liquid scintillation tubes

7. Add 4 ml of ready safe scintillation cocktail

8. Shake vigorously

9. Label tubes

10. Load tubes into liquid scintillation counter and start program 2

11. Results will automatically print

APPENDIX D: β ACTIN

FIG 8
β Actin

UNIVERSITY of NEW HAMPSHIRE

December 8, 2005

Tsang, Paul
Animal & Nutritional Sciences
Kendall Hall
Durham, NH 03824

IACUC #: 051103
Approval Date: 11/18/2005
Review Level: C
Project: Ovarian and Environmental Influences on Embryonic/Fetal Mortality in Ruminants

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the research potentially involves minor short-term pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics or other assessments.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this study. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:

1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building, 51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564